T H E S I S

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-:- by -:-

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ON THE OCCURRENCE OF ANTI-BODIES TO FERMENTS

IN THE NORMAL BLOOD SERUM,

WITH SPECIAL REFERENCE TO ANTI-TRYPSINE.

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The question of the presence, formation, mode of action and nature of the Anti-bodies in the serum, present naturally or formed after innoculation with specific substances either of a bacterial, toxin, or ferment nature is probably one of the most vital, certainly one of the most engrossing interest of the present day. At the same time it may be regarded as one of the most difficult subjects to grapple with; a subject where the points of vantage through which one may attempt an elucidation are strikingly few in number. On this account then, a contribution from the domain of physiology, that is in so far that the main subject of this thesis deals with the presence in the normal serum of an anti-body to a proteolytic ferment normally present in the organism, may be of some value. The research is all the more interesting as such anti-ferments differ very materially from those evolved by the injections of substances of bacterial origin, in that the degree of anti-ferment power developed after the injection of the enzyme into the organism

exceeds only in very slight degree that of the normal serum itself.

As I have just stated, the subject of my thesis is the presence of enzymic anti-bodies, with special reference to that of Trypsine, present in the normal blood serum. The fact that an anti-body to Trypsine exists has been known now for a considerable number of years, but the subject has, as far as my knowledge goes, never been fully worked out.

Probably the first direct reference, at any rate the earliest reference that I have been able to find, to the presence of an Anti-body, or at least of some substance present in normal horse serum which prevented full activity of a ferment, is that of RÖDÉN (1), when this observer found that the addition of a small quantity of blood serum reduced considerably the action of rennet. Many years later MORGENROTH (2) carried out a series of experiments with this same ferment and its anti-body. FULD and SPIRO (3) and later FULD (4) alone again dealt particularly with this ferment, as did also PUGLIESE & COGGI (5) and HAHN (6), the results in all cases fully bearing out the statement of RÖDÉN.

With regard to Pepsin, (which will be discussed in greater detail later,) SCHNAPPAUF (7) found that both

blood and blood serum markedly hindered or even completely prevented the action of this enzyme. BRIOT (8), PERIN(9) HAHN (l.c) PUGLIESE and COGGI (l.c.) and SACHS (10) have all dealt with this question in a more or less complete fashion. WEINLAND (11) also investigated the subject, but devoted his attention more particularly to the anti-body which he discovered to be present in the stomach wall. Anti-bodies have likewise been obtained in blood serum for the enzymes of the Spleen and of the blood itself by HEDIN (12), for Urease by MOLL (13), for Diastase by PUGLIESE and COGGI (l.c) and HAHN (l.c), for the proteolytic enzyme in yeast juice by HAHN (14), for Lipase by SCHÜTZE (15), and for Zymase by JACOBSOHN (46).

Turning now to the literature of the anti-tryptic substance with which I am more intimately concerned, the first definite notice of the power which normal serum possesses of hindering or of completely preventing the action of Trypsine was that of Hahn (l.c) in Germany and almost contemporaneously with the German publication that of PUGLIESE and COGGI in Italy. Several years earlier however, FERMI and PERNOSSI (16) had noticed that Trypsine rapidly disappeared after injection into the animal body. They likewise found that Trypsine was destroyed by the tissues in vitro, this result being probably due

to the serum content of the same. Since then the question has fairly frequently been discussed by various workers, ACHALME(17) CAMUS and GLEY (18) CHARRIN and LEVADITI (19) SIMNITZKI (20) and DELEZENNE (21), but in rather a cursory fashion. There still remain however, two papers of more recent origin in which a serious effort was made to get a little nearer to the elucidation of the question by attempting to isolate the anti-body or at least that fraction of the serum to which it appeared to be attached. The results obtained by the two investigators LANDSTEINER (22) and GLAESSNER (23) were so divergent that beyond the fact that both agreed in accepting the presence of an anti-body to trypsine, no definite conclusions could be drawn.

The earlier worker of the two, LANDSTEINER (l.c.) made a distinct step forward when he, for the first time, attempted the isolation of the anti-body by fractionation of the serum. His published results however give one very little information except that he believed the anti-action manifested by the serum to be intimately connected with the fraction known as the Albumine fraction, that is that fraction which is precipitated on full saturation of the serum with ammonium salts.

GLAESSNER on the other hand, whilst attempting to

repeat LANDSTEINER'S work, came to an entirely different conclusion as regards the fraction of serum to which the anti-action belonged. His results shew that this action was brought about by the use of the Euglobulin fraction, that is the fraction coming down on 1/3 saturation with ammonium salts.

In both cases Ammonium Sulphate was the salt employed for fractionation. As it is a very convenient salt to work with, I also have used it throughout the present investigation. Both LANDSTEINER and GLAESSNER used as the means of gauging the extent or degree of digestion METT'S Tubes (capillary glass tubes filled with coagulated proteid, as a rule boiled egg albumen), whereas during my experiments I employed an entirely different method which I shall presently describe in detail. This method was the one used by Dr. Hedin, to whom I am indebted for much kindness and advice, with great success in his splenic enzyme researches.

M E T H O D S.

With regard now to the technique of the experiments, I shall first deal with the mode of fractionating the serum. It was essentially that described by FREUND and

JOACHIM (24).

(1) FRACTIONATION OF THE SERUM.- The serum employed was that obtained from various species of animals, as a rule however ox blood was employed as it was the most easily obtained. The blood, after being received into a perfectly clean vessel direct from the animal, at the time of killing, was allowed to coagulate; the following day the serum was pipetted off and this again freed from any blood corpuscles present by means of the centrifuge. A measured quantity of this serum was taken and diluted with two or three times its volume of distilled water, and then cold saturated solution of Ammonium Sulphate was added up to 1/3 saturation, this resulting in the precipitation of the so-called Euglobulin. This precipitate was filtered off on the following day and the filtrate brought up, by the addition of more of the sulphate solution, to 1/2 saturation with the result that the fraction known as Pseudoglobulin was precipitated. This again was allowed to stand for 24 hours and then filtered off, the filtrate being then fully saturated by the addition of the requisite amount of solid dry Ammonium Sulphate which threw down the so-called Albumine fraction. The first two precipitates were redissolved in known volumes of distilled water and again precipitated with the proper volume of sulphate

solution. This re-precipitation was always carried out twice, sometimes even three times. As nothing was to be gained by repeated precipitations in the case of the Albumine fraction they were omitted. The final precipitates were dissolved in as little water as possible and transferred to parchment dialysers, and dialysed against running water, in some instances against distilled water, until all Ammonium Sulphate was practically removed. The solutions obtained from the dialysers were then brought up, when necessary, to the original volume of the serum employed either by means of distilled water or by 0·25% sodium carbonate solution.

(2) PREPARATION OF THE TRYPSINE SOLUTION.- Two or three Ox (or other, see later) Pancreases were taken, freed from as much fat and fibrous tissue as possible and then finely minced. To this mince was then added 2000 c.c. water and some 5 c.c. 20% Acetic Acid, and after addition of a small quantity of Toluol and Chloroform (to prevent putrefaction) the mixture was allowed to digest, in a large stoppered bottle, at 37° for two days, then filtered. The filtrate was returned to the incubator and allowed to digest until, on testing, practically no biuret reaction was given. It was then once more filtered, and the filtrate transferred to a dialyser in running water in order to free the enzyme

solution as completely as possible from its crystalline dialysable products (Tyrosine etc.) in order that the nitrogen figure of the ferment solution might be a low one. Solutions of ferments made in this way retain their activity for long periods, if kept in the ice chamber, although not quite as well as those solutions which contain more proteid matter.

(3) MATERIAL USED FOR DIGESTION.- In the majority of cases, particularly during my earlier experiments, the substance used as material for digestion was coagulated blood serum. In order to get quite accurate amounts of this, the fluid serum was pipetted off in the required amount into suitable digestion bottles, which were then placed for ½ an hour in a water bath heated to boiling point. Resulting coagulum was next cut up, still of course in the bottle, into cubes of as near one size as possible. In the later series however the material employed was a 3½% solution of Caseinogen in 0·25% Sodium Carbonate solution. This formed a very suitable medium, especially for quantitative work, as it shows very small differences in the degree of digestion.

(4) MODE OF ANALYSIS.- After the digests had remained in the incubator at 37° for the requisite time, a given

volume, that is an amount sufficient to precipitate all precipitable proteid, of Tannic acid solution was added.(+) After the mixture had remained standing over night to ensure perfect precipitation, it was filtered and a measured quantity of the filtrate used for carrying out the estimation of the Nitrogen present by Kjeldahl's method. In the following tables of results the figures given are the number of cubic centimetres of decinormal sulphuric acid ($\frac{N}{10}$ H_2SO_4) required to neutralise the ammonia distilled over. Titration was carried out with decinormal Soda solution, a solution of lacmoid being used as indicator.

The Tannic Acid solution was chosen as the best precipitant in a general way for such digestions where there is no mineral acid present (SEBELIEN (25). The solution can precipitate all substances higher than peptones, this means that in the filtrate there only appears the peptone and any lower products such as the mono or di-amino acids

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(+) Tannic Acid Solution.

Tannic Acid	100 grms.
Sodium Acetate	25 grms.
Sodium Chloride	75 grms.
Glacial Acetic Acid	50 c.c.m.
Distilled Water	..to 1000 c.c.m

which may have been formed in the course of digestion. In this way one gains a fair constant for the estimation of the degree of activity of the various solutions used. It gives far more accurate results than Mett's Tubes which depend for their results on the measurement of the amount of proteid digested out of the capillary.

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DEMONSTRATION OF THE PRESENCE OF AN ANTIBODY TO TRYPSINE IN NORMAL SERUM.

That there exists such a body as Anti-trypsine the figures, which I intend to present now (see Table I.) show, I trust, beyond all manner of doubt. Not only do they demonstrate the fact that serum itself possesses this anti-action, but they go further and support Landsteiner, as against Glaessner, in his contention that the anti-action is in connection with the so-called Albumine fraction. In this series to serve for purposes of comparison I have demonstrated (1) the use of boiled fraction, ($\frac{1}{2}$ hour at 100°) and (2) the results of using boiled trypsine solution (again $\frac{1}{2}$ hour at 100°). In the latter case it may be noted, as a matter of some interest, that the Euglobuline fraction shows a little more digestion than either of the other two fractions. This may be taken, I believe, as demonstrating the presence of the proteolytic enzyme found by HEDIN (l.c.) in the blood of the ox and attached to the Euglobuline fraction. It may be also

TABLE I.

Ppt. immed. with Tannic Acid (1) Sol. 5cc. of Filtrate for Analysis.

Control.

Medium.	Fraction.	Trypsine.	$\frac{N}{10}H_2SO_4$
5cc. C.S. (2)	5cc. Euglob. sol.	5cc.	0.4cc.
" "	" Pseudogl. sol.	"	0.4cc.
" "	" Albumine sol.	"	0.2cc.
" "	" Serum sol.	"	0.5cc.

Digest. 4 days at 37°.

	Medium.	Fraction.	Trypsine.	
A.	5cc. C.S.	5cc. Euglob. sol.	5cc.	6.8cc.
	" "	" Pseudogl. sol.	"	6.9cc.
	" "	" Albumin sol.	"	0.9cc.
	" "	" Serum sol.	"	1.1cc.
B.	5cc. C.S.	5cc. Eu. boiled	5cc.	7.9cc.
	" "	" Pseu. "	"	7.3cc.
	" "	" Alb. "	"	7.2cc.
C.	5cc. C.S.	5cc. Eu.	5cc. boiled	1.2cc.
	" "	" Pseu.	" "	0.30cc.
	" "	" Alb	"	0.35cc.

(1) Of course all digests of series are ppt. with same amount of acid. In all series given 10cc. acid was used.

(2) C.S. = Coagulated Serum.

worthy of note that in a number of instances one finds that the pseudo-globuline fraction possesses some slight anti-tryptic action, which however may be neglected (1) on account of the slight action, and (2) on account of the relatively gross method employed to fractionate the serum it can quite easily, owing to the irregularity of its appearence, be regarded as impurity, that is, that the fraction was not completely freed from Albumine.

That this anti-action is not exerted only against one variety of proteid is proved by the fact that it acts equally well whether the proteid be in a solid or a fluid state, for example the medium employed may be coagulated egg albumen (action here not quite so rapid as in case of coagulated blood serum) or boiled anti-body solution, or casein solution, etc. (See Table II.)

Of course the objection might be raised that the anti-action as demonstrated above might be more apparent then real, that digestion actually occurred but did not go so far as to be demonstrated by the Tannic acid method of precipitation which was employed. That such was not the case can be simply proved by carrying out a series of experiments where visual examination was the mode employed for observing the degree of digestion. (It may be noted here that as I mentioned before previous workers using

TABLE II.

A. Controls. Ppt.with Tannic Acid, 10cc.Filtrate for Analysis.

Antibody.	Digest Subst.	Trypsine.	0·25% Na_2CO_3	$\frac{N}{10}H_2SO_4$
5cc.Ser.Alb.	10cc.3½% Casein sol.	5cc.	-------	0·8cc.
-------	10cc.3½% Casein sol.	5cc.	5cc.	0·5cc.
5cc.Ser.Alb.	10cc.boiled Ser.Alb.	5cc.	-------	0·5cc.
-------	10cc.boiled Ser.Alb.	5cc.	5cc.	0·4cc.
5cc.Ser.Alb.	----------	5cc.	10cc.	0·25cc.

B. Digests. 20 hours at 37°.

Antibody.	Digest Subst.	Trypsine.	0·25% Na_2CO_3	$\frac{N}{10}H_2SO_4$
5cc.Ser.Alb.	10cc.3½% Casein sol.	5cc.	-------	2·0cc.
-------	10cc.3½% Casein sol.	5cc.	5cc.	11·9cc.
5cc.Ser.Alb.	10cc.boiled Ser.Alb.	5cc.	-------	0·6cc.
-------	10cc.boiled Ser.Alb.	5cc.	5cc.	3·0cc.
5cc.Ser.Alb.	----------	5cc.	10cc.	0·35cc.

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Mett's Tubes had come to like results. In this case the question of imperfect estimation by means of precipitation could not enter.)

Cubes of coagulated Egg Albumen were left for several days in contact with Trypsine and the various fractions. In every case the cubes in the digest containing the Albumine fraction retained more or less perfectly their sharp outline, those in the others being more or less digested. Another variation (see Table III.) of this experiment was made where the cubes of egg albumen of approximately equal size, were stained as homogeneously as possible with carmine. After the excess of the stain had been removed by thorough washing, the cubes, of course in weighed amounts, were placed in mixtures of the Trypsine and the various fractions as usual. The amount of stain set free after digestion of about a week's duration was estimated much after the method employed for Haemoglobin. The solution which I employed for diluting was 20% Acetic Acid as I found that this acid gave the brightest and sharpest colour result. In the first place to 1 c.c.m of the filtrates from the digests was added 1 c.c.m of the acid. The figures in the Table give the further additions of acid, in cubic centimetres, necessary before there was uniformity in colour.

TABLE III.

Digests at 37°.

					No.of cc.Acid added.
2½ grms.	C. Egg	Alb.	5cc.Euglob.	5cc.Tryp.	4cc.
"	" "	"	" Pseu.	" "	3cc.
"	" "	"	" Albu.	" "	1cc.
"	" "	"	"0.25% Na_2CO_3 "	"	3½cc.

Of course this result is at best a very crude one, as it must be remembered that a certain amount of diffusion would take place, but still it is of some interest, more especially as it bears out very satisfactorily the more elaborate Tannic Acid estimations.

Although the above sets of experiments were carried out with Trypsine obtained from Ox pancreas and with Ox blood serum, the results obtained with Trypsines and sera of other animals, horse, pig, etc., were equally satisfactory and conclusive. As I stated before the reason I used Ox serum and trypsine was owing to the fact that it was most easily obtained.

The fact that all sera and Trypsines gave like results naturally leads one to enquire if this reaction is specific, that is, does anti-action only occur when one uses serum and trypsine from the same animal or species. Glaessner in his paper answers, on what to me seems rather slender evidence, very decidedly in the affirmative. The results which I obtained, whilst investigating this question, were rather unsatisfactory and inconclusive. There was without doubt some slight "apparent" specific action (see Table IV.), but the result can be questioned very seriously as it is practically impossible to obtain either trypsines or sera of different animals of like degrees of

efficiency and concentration. In the following tables are given some of the results obtained. The tables IV-VI form a complete series, but as I have just stated it is impossible to appraise their true value. Unfortunately the specimen of serum albumine of pig's blood employed, was not very active and so rather mars Table V.

Table VI. offers a very good example in favour of my contention with regard to the specificity of the action. Here the Ox serum holds the Ox trypsine in check as it likewise does the pig trypsine; on the other hand, however, when we come to deal with the Ox trypsine in relation to the Pig's serum this latter seems unable to hinder so completely the action of the $\overset{ox}{\wedge}$ trypsine as it does the pig trypsine. If then the actions of the various sera and trypsines were specific, one would have expected a similar result when Pig Trypsine was used in conjunction with Ox serum to that of Ox Trypsine and Pig serum, whereas actually the result is slightly less than that with Ox Trypsine, viz. 0·7 to 0·75.

On these grounds then I venture to think that one may conclude that the evidence is rather against real specificity. This view is quite in keeping with the results obtained by MESNIL (26). Of course one may remember that MORGENROTH (27) among others has found that one

TABLE IV.

Ppt. immed. with Tannic Acid. 5cc. Filtrate for Analysis.

0x Serum - Pig Trypsine.

					$\frac{N}{10}$ H_2SO_4
A. Control.					
5cc. C.S.	x	5cc. 0x Eu.	x	5cc. Pig Trypsine.	0·5cc.
" "	x	5cc. 0x Ps.	x	5cc. Pig "	0·6cc.
" "	x	5cc. 0x Alb.	x	5cc. Pig "	0·5cc.
B. Digests.	after 3 days at 37°.				
5cc. C.S.	x	5cc. 0x Eu.	x	5cc. Pig Trypsine.	5·5cc.
" "	x	5cc. 0x Ps.	x	5cc. Pig "	5·9cc.
" "	x	5cc. 0x Alb.	x	5cc. Pig "	3·5cc.

* *

TABLE V.

A. Control. Ppt. with Tannic Acid. 5cc. Filtrate for Analysis.

Pig Serum - 0x Trypsine.

5cc. C.S.	x	5cc. Pig Eu.	x	5cc. 0x Trypsine.	0·3cc.
" "	x	5cc. Pig Ps.	x	5cc. 0x "	0·25cc.
" "	x	5cc. Pig Alb.	x	5cc. 0x "	0·3cc.
B. Digest.	3 days at 37°.				
5cc. C.S.	x	5cc. Pig Eu.*	x	5cc. 0x Trypsine	8·3cc.
" "	x	5cc. Pig Ps.	x	5cc. 0x "	8·1cc.
" "	x	5cc. Pig Alb.	x	5cc. 0x "	7·95cc.

* *

* Same Pig Serum fractions under same conditions but with Pig Trypsine

5cc. Pig Eu. + 5cc. Pig Trypsine $\frac{N}{10}$ H_2SO_4 5.4cc.

" " Ps " " " 5.4cc.

" " alb. " " " 2.8cc.

TABLE VI.

Ppt. with Tannic Acid. 5cc.Filtrate for Analysis.

A. Control.

Medium.	Fraction.	Trypsine.	$\frac{N}{10}H_2SO_4$
5cc.C.S.	5cc. Ox Ser.	5cc. Ox Tryp.	0·8cc.
" "	" Ox Ser.	" Pig Tryp.	0·5cc.
" "	Pig Ser.	" Ox Tryp.	0·5cc.
" "	" Pig Ser.	" Pig Tryp.	0·4cc.

B. Digest. 3 days at 37°.

5cc.C.S.	5cc.Ox Ser.	5cc.Ox Tryp.	0·75cc.
" "	" Ox Ser.	" Pig Tryp.	0·7cc.
" "	" Pig Ser.	" Ox Tryp.	1·4cc.
" "	" Pig Ser.	" Pig Tryp.	0·6cc.

can get an anti-substance produced (by artificial injection) against a vegetable rennet - Cyanarase - which is practically inactive when tested with rennet of animal origin. From a similar standpoint the experiments of GESSARD (28) with Tyrosinase are extremely interesting.

Owing to the insistent and dogmatic fashion in which Glaessner asserted his belief that "Anti-trypsine" was bound up with the Euglobulin fraction I made some investigation as to whether there might not be two anti-bodies for trypsine present in the serum, similar to the true and pseudo-anti-body to rennet found by KORSCHUN (29) in horse's serum.

To this end I prepared a solution of the globulins, obtained by the addition of a very small quantity of Acetic Acid to serum, which had been diluted with several times its volume of water. The resulting precipitate was carefully collected and thoroughly well washed with water. It was then dissolved in a small quantity of 0·25% sodium carbonate solution (Globulines from about 300 c.c.m serum dissolved in 100 c.c.m 0·25% soda). Digests were next prepared (see Table VII.) with and without casein and Globulines; globulines heated to 70° for ½ hour (in order to destroy the proteolytic ferment normally present in the serum (Hedin); and digests with and

TABLE VII.

A. Control. Ppt. immed. with Tannic Acid, 10cc. Filtrate for Analysis.

			$\frac{N}{10} H_2SO_4$
5cc. Globulin sol.	x 5cc. 0·25% Na_2CO_3	x 1cc. Trypsine	0·3cc.
" " "	x " sat. sol. Casein	x 1cc. "	0·4cc.
" sat. sol. Casein	x " 0·25% Na_2CO_3	x 1cc. "	0·35cc.

B. Digests. 4 days at 37°.

5cc. Globulin sol.	x 5cc. 0·25% Na_2CO_3	x 1cc. Trypsine	0·7cc.
" " "	x " sat. sol. Casein	x 1cc. "	5·4cc.
" sat. sol. Casein	x " 0·25% Na_2CO_3	x 1cc. "	5·0cc.
5cc. Globulin sol. heated $\frac{1}{2}$ hour at 70°.	x 5cc. 0·25% Na_2CO_3	x 1cc. Trypsine.	2·8cc.
5cc. Globulin sol. heated $\frac{1}{2}$ hour at 70°.	x 5cc. sat. sol. Casein	x 1cc. Trypsine	6·4cc.
5cc. Globulin sol. heated $\frac{1}{2}$ hour at 100°.	x 5cc. 0·25% Na_2CO_3	x 1cc. Trypsine	2·2cc.
5cc. Globulin sol. heated $\frac{1}{2}$ hour at 100°	x 5cc. sat. sol. Casein	x 1cc. "	6·3cc.

without casein along with globuline solution which had been heated to 100° for ½ an hour.

This table shows, I think, beyond all manner of doubt the fact that there is no anti-body to be detected, at least by the methods which I have employed, in the (Eu) globuline fraction. This is very definitely shewn when one notes that the amount of digestion of casein alone = 5 c.c. of $\frac{N}{10}$ H_2SO_4 whilst when both Casein and Globuline solution are present together it equals 5·4 c.c. in the unheated, and in case where Globuline heated to 70° it amounts to 6·4 c.c. $\frac{N}{10}$ H_2SO_4 This table, and especially the figures just quoted (see also Table I.) bear out OPPENHEIMER and ARON'S (30) statement that the Globulines in their native state, that is unheated, normal state, are difficult to attack by means of enzymes. They say in the course of their work that: "Man könnte deshalb auf die Vermutung kommen dasz es ausschliesslich die Globuline sind, die vom Tyypsin nicht angegriffen werden."

Whilst on this subject it is interesting to note the results obtained from the application of some observations of MOLL (31) to the effect that one can convert the serum Albumine into serum Globuline by the action of heat. If this change really took place to any marked degree it was thought that it might offer a clue as to whether the anti-

action was simply dependent upon the serum albumine itself, or whether it was quite a separate body merely precipitated in conjunction with the albumine fraction, as if the anti-action were independent of the albumine, absolutely and relatively it would remain practically unaltered, whereas if it were part and parcel of the albumine it would be diminished both absolutely and relatively.

At all events I carried out the experiments, preparing the serum according to Moll's directions, but the results were absolutely negative as in both heated and unheated fractions the results were practically the same.

Again one thought, judging from the results of Martin and Cherry, Calmette, Lamb, etc, with Venin and antivenin that (1) there might be some difference in the degree and activity of the anti-body depending on whether the ferment and the anti-body had been in contact before the addition of the Proteid for digestion, and (2) that the time they - the ferment and anti-body - remained in contact before the addition might affect the ultimate result.

The figures obtained however, were quite negative as may be seen from the following table (see Table VIII.). It is from these and other similar series of figures where

TABLE VIII.

A. Control. Ppt. immed. with Tannic Acid 10cc. Filtrate for Analysis.

			$\frac{N}{10}$ H_2SO_4
5cc. Trypsine.	5cc. Ser. Alb. sol.	10cc. 0·25% Na_2CO_3	0·25cc.
5cc. "	5cc. Ser. Alb. sol.	10cc. 3½% Casein sol.	0·8cc.
5cc. "	5cc. 0·25% Na_2CO_3	10cc. 3½% Casein sol.	0.5cc.
5cc. "	5cc. Ser. Alb. sol	10cc. boiled Ser. Alb.	0.5cc.
5cc. "	5cc. 0·25% Na_2CO_3	10cc. boiled Ser. Alb.	0.4cc

B. Digests. 20½ hours at 37°.

	5cc. Trypsine	5cc. Ser. Alb. sol.	10cc. 0.25% Na_2CO_3	0.35cc.
α	5cc. "	5cc. Ser. Alb. sol.	10cc. 3½% Casein sol.	2.0cc.
	5cc. "	5cc. 0.25% Na_2CO_3	10cc. 3½% Casein sol.	11.9cc.
β	5cc. "	5cc. Ser. Alb. sol.	10cc. boiled Ser. Alb.	0.6cc.
	5cc. "	5cc. 0.25% Na_2CO_3	10cc. boiled Ser. Alb.	3.0cc.
γ	5cc. "	10cc. 3½% Casein sol.	5cc. Ser. Alb. sol.	2.1cc.
δ	5cc. "	10cc. boiled Ser. Alb.	5cc. Ser. Alb. sol.	0.65cc.

NOTE.- In (α) and (β) Trypsine and Antibody (Serum Albumine) were in contact 5 minutes before the addition of the other proteid.

In (γ) and (δ) Trypsine and proteid were in contact 5 minutes before the addition of the Antibody.

Serum Albumine - Antibody - was heated, in cases where inactive antibody was used, to 100° for 40 mins. on the water bath.

contact remained up to 30 minutes quite evident then that the union, be it chemical combination or physical adsorption, is not affected by the time in which they are in contact.

With regard to the Temperature at which the anti-body is destroyed it seems to depend to some degree on whether one is dealing with the isolated anti-body, that is the serum Albumine fraction, or with the whole untreated serum. Another factor which apparently plays rather an important part under this head is, whether one has used distilled water or 0·25% Sod.Carb.sol. to dissolve the anti-body in. The presence of the alkali, as my experiments show, exercises rather a harmful influence in so far that it causes the Anti-action to be suspended at a lower temperature than is the case where the alkali is absent. This deleterious action of the alkali is quite as marked when serum itself, instead of the isolated fraction, is employed as Anti-ferment.

The first table given (see Table IX.) illustrates the effect of heat upon the isolated anti-body at the same time demonstrating the destructive action of the carbonate solution when that is present.

In each case the antibody was kept in the water, both at the given temperature for ½ an hour, and 10 c.c. of the

TABLE IX.

A. Control. Ppt. immed. with Tannic Acid. 10cc. Filtrate for Analysis.

Medium.	Serum Albumine.	Water.	Trypsine.	$\frac{N}{10}$ H_2SO_4
5cc. C.S.	5cc. Active	5cc.	5cc.	0·5cc.

I.

B. Digests. 4 days at 37°.

5cc. C.S.	5cc. Active	5cc.	5cc.	0·85cc.
" "	" 45°	"	"	0·8cc.
" "	" 50°	"	"	0·85cc.
" "	" 55°	"	"	1·2cc.
" "	" 60°	"	"	11·0cc.
" "	" 100°	"	"	12·75cc.

II.

5cc. C.S.	x	5cc. Ser. Alb.	Active	x	5cc. 0·25%	Na_2CO_3	x	5cc. Tryp.	0·8cc.
" "	x	" "	" 45°	x	"	"	"	"	x	"	"	0·75cc.
" "	x	" "	" 50°	x	"	"	"	"	x	"	"	0·8cc.
" "	x	" "	" 55°	x	"	"	"	"	x	"	"	4·05cc.
" "	x	" "	" 60°	x	"	"	"	"	x	"	"	13·5cc.
" "	x	" "	" 100°	x	"	"	"	"	x	"	"	13·4cc.

filtrate after precipitation with Tannic Acid was used for the examination.

In the case of Serum itself, the same marked difference is shewn, as may be seen from the following table, (see Table X.).

From these figures then it may be concluded that the anti-body is destroyed by heating for ½ an hour at a temperature between 60° - 65° in the case of the "whole" serum, and between 55° and 60° for the isolated fraction when alkali is present. When water is used the temperature in the case of the isolated fraction is apparently about the same as with alkali, but for the serum itself it is somewhat higher, viz. 65°-70°.

These figures agree very well with those of HAHN (l.c) and DELEZENNE (l.c.)

As to the effect of room temperature on the anti-trypsine it was found that solutions of the anti-body retain at this temperature a marked degree of anti-action for quite lengthy periods, both when in isolated form and as serum. When kept in the cold room the rate of destruction progresses, as would be expected, much more slowly; even after several months active samples can be obtained. The results which MORGENROTH (l.c.) obtained when working with Anti-rennin differed markedly from the above, as he

TABLE X.

I.

A. Control. Ppt. immed. with Tannic Acid. 10cc. Filtrate for Analysis.

Medium.	Serum.	Water.	Trypsine.	$\frac{N}{10}$ H_2SO_4
5cc. C.S.	5cc. Active	5cc.	5cc.	1·1cc.

B. Digests. $2\frac{1}{2}$ days at 37°.

5cc. C.S.	5cc. Active	5cc.	5cc.	1·7cc.
" "	" 55°	"	"	1·65cc.
" "	" 60°	"	"	1·6cc.
" "	" 65°	"	"	1·7cc.
" "	" 70°	"	"	2·0cc.
" "	" 100°	"	"	15·5cc.

II.

A. Control.

Medium.	Serum.	0·25% Na_2CO_3	Trypsine.	
5cc. C.S.	5cc. Active	5cc.	5cc.	0·9cc.

B. Digests. $2\frac{1}{2}$ days at 37°.

5cc. C.S.	5cc. Active	5cc.	5cc.	0·95cc.
" "	" 55°	"	"	0·9cc.
" "	" 60°	"	"	8·2cc.
" "	" 65°	"	"	61·1cc.
" "	" 70°	"	"	18·1cc.
" "	" 100°	"	"	20·5cc.

NOTE.- In all cases where the Serum was heated, it was kept at given temperature in water bath for $\frac{1}{2}$ an hour.

found that this anti-body deteriorated rapidly even when kept in the cold room.

The effect of drying has likewise been tried (see Table XI.). The anti-body precipitated by Ammonium Sulphate was allowed to dry on a filter at room temperature, the presence of the Ammonium Sulphate preventing putrefaction. After the mass was completely dried it was scraped off the filter paper and finely powdered. A portion of this powder was dissolved up in water and transferred to a dialyser in order to get rid of the sulphate. When the solution was free from the salt, digests were prepared with the following result:-

TABLE XI.

A. Control. Ppt. immediate with Tannic Acid 10 c.c. filtrate.

5 c.c.C.S.x 5 c.c.Dialy-sed "Dry" Antibody x 5 c.c.Trypsin = 0·7cc $\frac{N}{10}$ H_2SO_4

B. Ppt. after 4 days digestion at 37°

5 c.c.C.S.x 5 c.c.Dial."Dry" Antibody x 5 c.c.Trypsin = 1·1cc.

" " x " " " " x " " = 6·8cc.

" heated ½ hour at 100°.

These figures show unmistakeably that the property of hindering the action of the enzyme had been retained

by the dry albumine.

Dialysis has not been found during the course of this research, to cause much diminution of the activity of the solutions of the anti-body. It is not maintained of course, that the treatment does not have some deleterious effect, but it is only on very rare occasions that one found that a serum which possessed anti-action towards the ferment was when fractionated quite without action. Such cases have occurred but the cause remained a mystery as the methods were those usually employed with excellent results.

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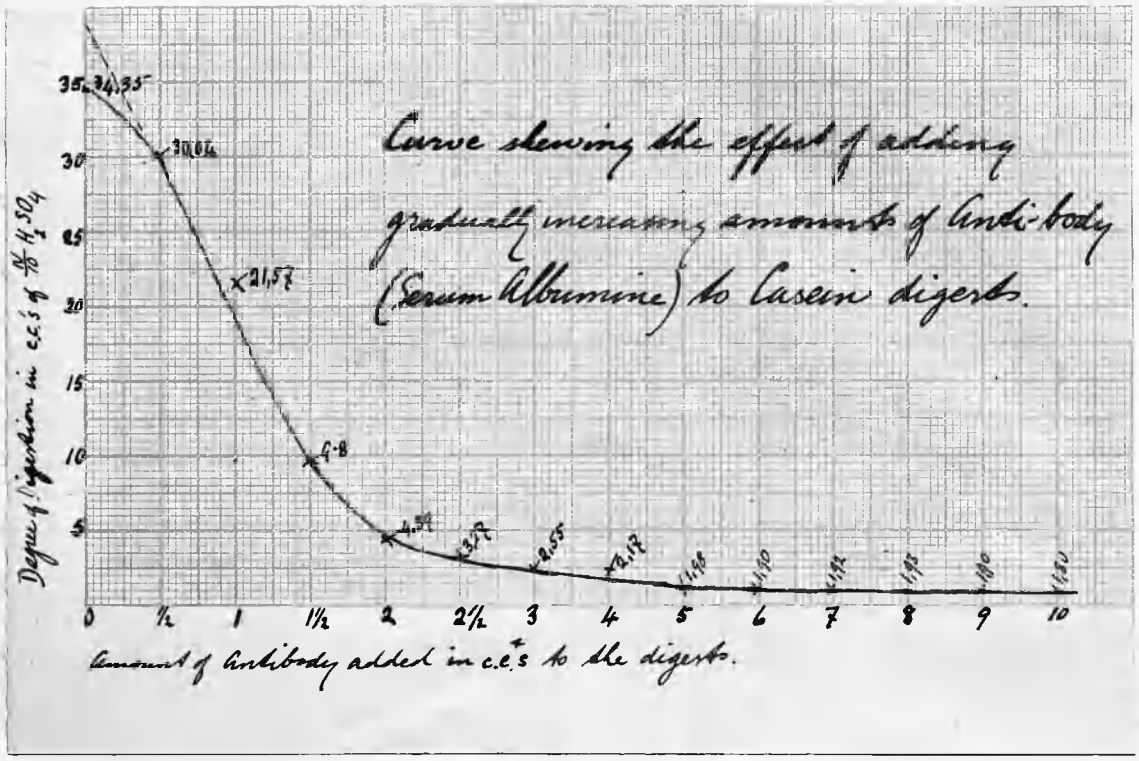
Lastly I should like to refer very briefly to some extremely interesting results which were obtained on working quantitatively with Trypsine and its anti-body, the more so as since the work has been in progress - it is not yet finished - Dr. Madsen referred to some work he had been carrying out with another anti-ferment, viz. Anti-rennet. Madsen found that this obeyed the law of mass action of Guldberg and Waage quite as well as did the anti-lysin and anti-toxin sera. As the work stands at present it is not definitely decided whether anti-trypsine obeys the simple formula.


Curve shewing the effect of adding graduall increasing amounts of Anti-body (Serum Albumine) to Casein digests.
Degree of digestion in c.c.s of N/10 H2SO4
Amount of Antibody added in c.c.s to the digests.
35
30
25
20
15
10
5
0
½
1
1½
2
2½
3
4
5
6
7
8
9
10
34,35
30,04
21,57
6·8
4·5
3·15
2·55
2·12
1·60
1·50
1·32
1·15
1·00
1·50

$$\frac{\text{Free Antibody}}{\text{Vol.}} \times \frac{\text{Free Trypsin}}{\text{Vol.}} = K \frac{(\text{Antibody Trypsin})}{\text{Vol.}}$$

or the one where two molecules are formed

$$\frac{\text{Free Antibody}}{\text{Vol.}} \times \frac{\text{Free Trypsin}}{\text{Vol.}} = K \frac{(\text{Antibody Trypsin})^2}{\text{Vol.}}$$

The first results which I obtained rather pointed to the latter formula whereas later figures obtained by Hedin suggested the first formula as being the correct one. As much larger quantities of material were used, thus reducing the experimental error, the later results will probably turn out to be right.

As to the why and wherefore of the presence of an anti-body to trypsine in the normal blood serum I do not believe that one requires to search far for an explanation.

As is well known now from the study of the toxines and their anti-toxines, the organism into which the toxine has been introduced sets about protecting itself with the resulting formation of the anti-toxines. The resemblance between toxines and enzymes is being rapidly shewn to be a very great one, both enzyme and toxine are active in the smallest of quantities, both are exceedingly sensitive to physical and chemical agents, both possess the property of being absorbed by finely divided precipitates etc.

H. Buchner drew attention to the points of resemblance between his alexines and ferments; Ehrlich and Morgenroth in various papers have also commented on this subject; see also KORSCHUN (32). An extremely good summary of the present state of our knowledge on this subject is given by Oppenheimer in the last edition of his book on the Ferments.

That ferments possess very toxic properties was shewn conclusively by HILDEBRANDT (33) many years ago, therefore it is not a matter for surprise if the organism having absorbed a substance with toxic properties did not elaborate some protective substance in the shape of an anti-body. That the digestive ferments can be absorbed into the blood stream has been very definitely proved in the case of pepsin by the experiments of Delezenne and Frouin quoted by METCHNIKOFF in his L'Immunité, p.70., and by MATTHES (34), who extirpated the stomach and found that pepsin or an enzyme behaving in all ways like that ferment previously present in the urine, disappeared from that fluid. FROUIN (35) quite recently has proved that the Pepsin is really absorbed from the stomach by the administration of large quantities of pepsin to dogs, whose stomachs had been completely removed, but without any of the ferment appearing in the urine.

That the non-activity of the pepsin which must have reached the kidneys for excretion via the blood stream, is due to the presence of an anti-body and not simply due to the destruction of the ferment by the alkalinity of the blood is shewn by the fact that the ferment in the urine is quite readily extracted in an active form. BRIOT (l.c.) and PERIN (l.c.) have dealt with this question. If one injects large enough quantities of the ferment as demonstrated by FERMI and PERNOSSI (l.c.) sufficient to overcome the normal anti-ferment action of the serum, one obtains an increased excretion with the urine. One may naturally wonder at first sight what the use of an anti-ferment to pepsin might be when the enzyme is circulating in an alkaline medium, in which reaction it will not digest in vitro. If, as SCHNAPPAUF (l.c.) held, the ferment or at least a portion of it was absorbed in the proferment state the presence of a protective agent is required as LANGLEY (36) found that pepsin could be liberated from the proferment by the action of weak alkalis. If it be excreted by the kidneys in the proferment state the active ferment would be set free by the acid reaction of the urine.

If one accepts the above evidence then as proof of the absorption and excretion of Pepsin one may likewise

accept the idea that Trypsine is also absorbed. It is however, much more difficult to offer proofs of this in the shape of evidence from the urine. Undoubtedly there is an enzyme, which acts in many ways like Trypsine, present in the urine. (In another paper I have worked at this question, especially as regards the products formed after prolonged digestion of fibrin by means of this ferment. At the end of this paper will be found a short synopsis of the results.) FERMI and PERNOSSI (l.c.) obtained similar results as with pepsin, on injection of large quantities of trypsine, i.e. when they injected the enzyme in sufficient quantity they state that it appears in the urine.

One naturally thinks that a much more active anti-ferment could be produced by immunising an animal by repeated injections of the enzyme. This procedure as a matter of fact has been carried out for Rennet by MORGENROTH (l.c.), for Pepsin by SACHS (l.c.), and by ACHALME (l.c.), LANDSTEINER (l.c.) and DEAN (37) for Trypsine. All managed to obtain anti-ferments of increased power over those of the normal serum with the exception of Landsteiner. In every case however, only a very low grade of immunity could be obtained, results being in no way comparable to the production of anti-toxin. The explanation offered by Ehrlich and Morgenroth to account for

the failure to obtain an anti-rennet of any marked power is quite capable of application to the case of trypsine as both are normal components of the animal organism. On injection the trypsine seizes on specific side chains already present, with which it enters into firm combination. To compensate for this seizure new side chains are elaborated, these forming the Anti-trypsin. From this point on, however, there is a marked difference from the formation of Anti-toxin after injections of Toxin, as in this latter case the anti-toxin accumulates in the blood in large amount, there being nothing with which it may combine, whereas in the case of ferment injections the anti-ferment formed is neutralised by the presence of the enzyme in the body, thus preventing at all times any great excess of anti-body free in the blood.

To quote MORGENROTH (l.c.) with regard to the question of Anti-rennet. He says "If one calculate, under the most favourable conditions, the quantity of Anti-rennin which is present in the total amount of the blood, after the injection of, say 6 grams of the ferment, one finds that the same is only sufficient to neutralise about 3-4 grams of enzyme."

The results which DEAN (l.c.) obtained with Trypsine are entirely comparable with those obtained by Morgenroth.

Dean only managed to raise the activity of his anti-body some eight times.

It must be borne in mind however if one utilises Ehrlich and Morgenroth's theory to explain the low degree of anti-trypsine formation that BAYLISS and STARLING (39) have proved that Trypsine is not excreted as such, therefore it cannot be in the form of trypsine in the gland. These workers have shewn that Trypsine is a third substance elaborated by the action of entero-kinase on trypsinogen, and at the same time they definitely state that the only place where they could find entero-kinase was in the intestinal mucous membrane. Then the trypsine which is required by Ehrlich and Morgenroth's theory to neutralise the newly formed anti-body and thus prevent its accumulation, must either be absorbed directly from the intestinal canal, or else trypsinogen is likewise capable of neutralising the antiferment. Against the absorption from the intestine is the fact of the anti-action of the intestinal walls themselves, which would be capable one would think, of preventing any absorption. Of course the explanation may lie in the fact that Delezenne's statement, although it has not been satisfactorily corroborated, concerning the wide distribution of entero-kinase is correct, then the ferment would be taken up as trypsinogen,

in the same way that SCHNAPPAUF (l.c.) stated that part at least of the pepsin is absorbed, and having been activated, perhaps by means of the leucocytes, which Delezenne declared to contain entero-kinase, serve to neutralise the anti-body formed. Whatever the theoretical explanation may be, the experimental fact remains that one never obtains an extremely active Anti-ferment.

It may be mentioned that DELEZENNE (l.c.) maintains that this anti-action is not an anti-tryptic one but that it is anti-entero-kinase or anti-kinasique, as he calls it. Quite recently too, ASCOLI and BEZZOLA (38) took up this question and they finally reached the conclusion that the anti-body acted both anti-tryptic and anti-kinasique. Whatever were the nature of the solutions of trypsine and anti-body Delezenne was working with, I can firmly attest that the anti-body I had was really anti-trypsine as on the addition of a solution of entero-kinase to my solution of trypsine there was no increase in its activity as there would have been had any trypsinogen been present to be broken up or set free by the entero-kinase. Again in favour of the action being really an anti-tryptic one and not anti-kinasique is the fact that entero-kinase is extremely labile, especially in the presence of trypsin. BAYLISS and STARLING (39). Of course I do not for one

moment deny the possibility of an anti-entero-kinase also being present in the serum, in fact all evidence seems rather to point to the existence of such a substance.

The explanation of Delezenne's statement is that he believes that trypsine is not a definite substance as Bayliss and Starling maintain, but that it is merely a mixture of trypsinogen and entero-kinase, therefore if he could prove that the anti-body was "anti-kinasique" and not anti-tryptic it would be valuable evidence in favour of his theory.

The question of the fraction of serum with which the anti-action is connected, is extremely interesting, more especially as it is in the case of Anti-trypsine the albumine one.

On the whole one would have thought that Glaessner was right when he stated that the anti-tryptic action was in connection with the so-called Euglobulin fraction as most evidence goes to show that it is the globuline portion of the serum which is the more resistant (Oppenheimer and Aron, etc.). In favour of such a view also is the work which has been done on the immune sera, for instance that of PICK (40) where he found that the Diphtheria anti-toxine in the case of the Horse was in connection with the Pseudo-globuline, and in the Goat with the

Euglobuline fraction.

On the other hand VERNON (41) in a recent paper has shewn that albumine can resist trypsine quite as effectually as globuline. JOACHIM (42) also in a paper published a short time ago quotes results obtained by EMMERICH and TSUBOI (43) where these observers found that in animals immunised against Swine fever and Pneumonia, as the degree of immunity rose the proportion of globulines in the blood fell whilst albumine increased in amount, this result rather pointing to the view that in the case of these diseases at least the anti-bodies bore some relation to the Albumine fraction.

As to the real mode of action between the ferment and its anti-body we are still in the dark. I am at present carrying out some experiments to try and obtain some evidence as to whether anti-trypsine as such exists, or whether the action merely is dependent on the albumine molecule by attempting to obtain an Anti-Anti-trypsine by injection of anti-trypsine into animals.

Whether the action is one of chemical combination between the Trypsine and the Albumine, or whether it can be explained on the physical ground that the albumine adsorbs the ferment and thus throws it out of action, has not yet been determined. Charrin and Levaditi suggested that the

organism is chiefly protected from the action of trypsine simply owing to the fact that none, or only a very small amount of the enzyme was absorbed after excretion into the lumen of the gut. Fermi, on the other hand, came to the conclusion that the ferment could not attack living tissue and thus protection was ensured.

In this connection the experiments of MATTHES (44) are of interest as he found that trypsine was quite powerless against living fresh red blood corpuscles. Fuld and Spiro formerly held the view that the anti-action in the case of rennet at least, depended on alterations of the amount of salts present in the anti-body. Recently however, they have declared that their theory only plays a secondary part.

It is quite possible that the configuration of the albumine molecule may play a very important part; it may be analogous to the resistance SCHWARZ (45) found in aldehyde combinations of proteid to the action of trypsine.

It is quite conceivable that in the unaltered, i.e. the unheated, normal, albumine molecule there may be a lack of points of attack for the enzyme, thus it - the albumine - is itself protected, and at the same time it may be capable of engaging the zymophore group of the

trypsine molecule in such a way as to render the ferment inactive towards other proteids which may be present.

CONCLUSIONS (of Special Part.)

1. There exists in normal serum an anti-body to Trypsine.
2. This anti-action is found in connection with the so-called albumine fraction, i.e. the fraction coming down after full saturation with Ammonium Sulphate.
3. This anti-action was found in all varieties of sera examined (Horse, Pig, Ox, etc.)
4. This anti-action is effective with all varieties of proteid, whether in solid (as coagulated serum) or fluid (as solution of casein) form, i.e. the presence of the anti-body prevents the action of the ferment on these proteids.
5. The specificity, i.e. whether the serum of one species protects against the trypsin of another species, of the anti-action is doubtful. The variations in action obtained are apparently dependent upon quantitative and qualitative differences, rather than on specific.
6. The rate of combination between the anti-body and the ferment appears to be rapid.

7. The isolated anti-body - the albumine fraction - is rapidly injured by heating, especially in the presence of alkali. Action is much reduced by heating $\frac{1}{2}$ an hour at 55^{o}.

8. The active serum itself is somewhat more resistant. Ill effect shown first about 60^{o} after $\frac{1}{2}$ hour's heating.

9. The solutions of anti-body do not deteriorate very rapidly, even at room temperature.

10. Dried anti-body retains its anti-ferment power very well.

11. Dialysis as a rule has no apparent deleterious effect.

12. Trypsine-anti-trypsine combinations apparently obey the law of mass action.

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A P P E N D I X.

The products which I obtained after 7 months digestion of fibrine by the enzyme acting in an alkaline medium, obtained from the urine by precipitation with casein, resembled those, with one exception, usually obtained by the action of trypsine itself. They were Histidine, Lysine, Tyrosine, Leucine, Alanine, Amido-valerianic Acid, and α-Pyrrolidinecarboxylic acid, Glutamic Acid, Ammonia and Phenyl-alanine. Instead however, of getting the active form of Arginine, I obtained the inactive modification, which substance has only been isolated once previously.

The paper is published in extenso in Salkowski's Festschrift. Oct. 1904.

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